AEM Accepts, published online ahead of print on 3 August 2007
Appl. Environ. Microbiol. doi:10.1128/AEM.00803-07

Running Title: SALMONELLA SEROVAR ENTERITIDIS IN FLIES FROM INFECTED HENS

Isolation of *Salmonella enterica* serovar Enteritidis from House Flies (*Musca domestica*) Found in Rooms Containing *Salmonella* serovar Enteritidis-challenged Hens

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ABSTRACT

House flies (*Musca domestica*) released into rooms containing hens challenged with *Salmonella enterica* serovar Enteritidis (*Salmonella* serovar Enteritidis) rapidly became contaminated with *Salmonella* serovar Enteritidis. Forty to 50% of the flies were contaminated at 48 hours which increased to 50-70% at 4 and 7 days post exposure and then decreased to 30% at day 15. Initial attempts at recovering surface organisms for culture using an aqueous rinse was largely unsuccessful while culture of internal contents readily recovered *Salmonella* serovar Enteritidis. However, when 0.5% detergent was incorporated into the rinse, high bacterial recoveries were observed from both external and internal culture regimens, indicating equal distribution of the organism on and in the fly and a tighter interaction of the organism with the host than previously thought. *Salmonella* serovar Enteritidis was isolated routinely from the fly gut, on rare occasion from the crop, and never from the salivary gland. Feeding contaminated flies to hens resulted in gut colonization of a third of the birds but release of contaminated flies in a room containing previously unchallenged hens failed to result in colonization of any of the subject birds. These results indicate that flies exposed to an environment containing *Salmonella* serovar Enteritidis can become colonized with the organism and might serve as a source of *Salmonella* serovar Enteritidis transmission within a flock situation.

Introduction

Salmonella enterica serovar Enteritidis (*Salmonella* serovar Enteritidis) remains a serious human food-borne threat within the U.S. and overseas (6, 30). Poultry and their products constitute a significant proportion of the sources implicated in food-borne *Salmonella* outbreaks (30, 34, 36) prompting more rigorous focus by regulatory agencies and industry to implement measures to reduce incidence of these problem organisms on-farm and during processing. Many risk factors for exacerbating *Salmonella* infection in flocks have been identified on the farm and animal vectors, both vertebrate and invertebrate, have been implicated in this role (13, 14, 37). Insects have long been associated with pathogen spread in human disease outbreaks (1, 9) and similar observations were reported for poultry. Cockroaches (24), beetles (25, 33), and flies (10, 25, 29) recovered from poultry houses have all been reported to harbor *Salmonella* or other human pathogens and chicken-to-chicken transmission of these organisms has also been observed (32).

Flies comprise a large and complex fauna of arthropods with world-wide distribution. Because of their intimate relationship with decaying matter, garbage, and feces, flies have long been associated with potential disease spread. Olsen (28) lists 47 fly species where *Escherichia coli*, an indicator of fecal contamination, had been isolated. Seventeen of these were found to carry the human pathogens *Salmonella* or *Shigella* and, of these, 14 were considered "communicative", meaning they moved between contaminated environments and interacted with man (8, 28). The house fly (*Musca domestica*) was a prominent member of this group and received the most citations regarding contamination with human pathogens. With regards to poultry, while a number of studies examined flies as a carrier of *Salmonella* contamination, no work has focused on the kinetics of fly colonization with *Salmonella* upon exposure to a contaminated environment and where this organism resides on/in its arthropod host. The current study examined the time frame of fly contamination upon release into a room containing hens infected with *Salmonella* serovar Enteritidis, the location of the organism on/in the fly, and whether these contaminated flies could transmit *Salmonella* to naive non stressed and stressed hens.

MATERIALS AND METHODS

Chickens. Single-Comb White Leghorn chickens, >60 weeks of age, were obtained from the specific-pathogen-free flock maintained at the Southeast Poultry Research Laboratory (SEPRL), Athens, GA. Twenty-six, 24, and 26 hens were used in Expt. 1, 2, and 3, respectively. The hens were transferred to individual adjacent laying cages in an environmentally controlled BSL-2 building at SEPRL and allowed to acclimate 7-14 days. The hens were fed layer ration *ad libitum* throughout the experiment duration. To assure that the hens were *Salmonella*-free, the individuals were screened for *Salmonella* prior to the commencement of experiments by enriching 1 g feces in 9 ml Rappaport-Vassiliadis (RV) enrichment medium (Oxoid Inc, Basingstoke, England), incubating overnight at 37°C, and plating 100 µl of the broth onto XLT4 agar (Remel, Lenexa, Kansas). *Salmonella* was not detected. The studies were approved by and conducted under the guidelines of the SEPRL Institutional Animal Care and Use Committee.

Infection. Frozen stocks of nalidixic-acid-resistant *Salmonella* serovar Enteritidis (strain SE89-8312), were maintained at -20°C. For each experiment, three days prior to infection, the *Salmonella* serovar Enteritidis was thawed and cultured onto nutrient agar (Difco/Becton Dickinson Microbiology Systems, Sparks Maryland) at 37°C for 18-24 h. An individual colony of the *Salmonella* serovar Enteritidis was re-cultured onto nutrient agar and incubated at 37°C for 18-24 h. A 10 ml tube of tryptic soy broth (Difco) was then inoculated with isolated colonies from the nutrient agar plate and incubated overnight at 37°C. The *Salmonella* serovar Enteritidis broth culture was diluted 10^{-2} in sterile saline and each bird received a dose of 1 ml *per os* (9 X 10^6, 5.6 X 10^6, and 3 X 10^6 *Salmonella* serovar Enteritidis in experiments 1, 2, and 3, respectively). At the time of challenge, 6 food-grade 23- X 28- cm polystyrene trays (Genpack Corp., Glens Falls, NY) were placed on the floor beneath the cages in each room and served as the site for sampling environmental fecal *Salmonella* serovar Enteritidis levels. On testing days, four 1-g fecal samples were obtained from individual trays and placed into separate plastic stomacher bags. Sites were randomly selected for each day. The samples were diluted 10-fold in RV broth and then plated onto brilliant green agar containing 20 µg/ml of nalidixic acid and novobiocin (BGNN) using an Autoplate 4000 automatic dilution/plating system (Spiral Biotech, Norwood, MA). Following 24 h incubation at 37°C, *Salmonella* serovar Enteritidis counts of the plates were made using a QCount plate reader (Spiral Biotech). For the samples with no detectable *Salmonella* serovar Enteritidis growth on the plates, the RV enrichment for that sample was plated onto BGNN, these plates were incubated a further 24 hours at 37°C and evaluated for the presence of *Salmonella* serovar Enteritidis.

Flies and Sampling. House flies (*Musca domestica*) were received as pupae, approximately two days prior to fly emergence, from the USDA/ARS Center for Medical and Veterinary Entomology, Gainesville, FL. The pupae, approximately10^4, were received in a cardboard container and the container, with the lid removed, was placed into the bird room. The placement occurred 3 days prior to hen challenge to ensure maximum fly density at the time the birds received the *Salmonella* serovar Enteritidis. A second batch of 10^4 pupae was placed into the rooms 7 d later to replenish the fly population. In order to encourage natural visitation by the flies to the birds and the food and feces, no additional food was provided to the flies. On day of sample, one QuikStrike fly abatement strip (Wellmark International, Schaumburg, IL), was placed on a fresh polystryene tray on the floor of each room. The flies attracted to the bait on the strip consumed a portion of it and rapidly succumbed to the nithiazine toxicant. The dead flies were retrieved using sterile forceps, one forcep/fly, and deposited into individual zip-sealable plastic bags. Flies were collected individually immediately after knockdown to eliminate cross-contamination after knockdown. Twenty flies/room were collected on each day of sample and were transported to the laboratory for culture. Each bag received one ml RV broth and the flies were thoroughly crushed and macerated into the broth using thumb and forefinger. One hundred µl of sample was then spread-plated onto BGNN. The plates and the bags containing the remainder of the fly/RV mix were incubated for 24 h at 37 ^{o}C. Following incubation, the BGNN plates were evaluated and *Salmonella* serovar Enteritidis counts were ascertained. For the samples with no detectable *Salmonella* serovar Enteritidis growth on the plates, the RV enrichment for that sample was plated onto BGNN, these plates were incubated a further 24 hours at 37^{o}C and evaluated for the presence of *Salmonella* serovar Enteritidis. Samples with no growth on the direct plating, but positive in the RV enrichment were given an arbitrary count of 9 (1 below the theoretical detection limit) and samples with no growth in either the direct plating or the RV enrichment were given a count of 0.

***Salmonella* serovar Enteritidis localization on/in the fly.** Three trials were performed to determine location of *Salmonella* serovar Enteritidis on/in the fly. In trial 1, 7 flies were collected on each of two separate days from experiment-2 hens into individual sterile tubes. One ml of RV broth was added to each tube and the tubes vortexed at maximum speed for 30 s (=Rinsed). Each fly was then placed into a tube containing 1 ml of bleach (800 ppm chlorine), vortexed for 30 s, and then placed into a tube containing 1 ml sterile distilled water and vortexed a further 30 s. The fly was removed from the water and added to a tube containing 1 ml RV and vortexed a further 30 s (=Clean). The flies were then transferred individually

to a fresh 1 ml RV contained in a zip-sealable plastic bag and crushed and macerated between thumb and forefinger (=Inside). All samples were incubated 37 °C for 24h and then plated onto BGNN. Following a further 24h incubation at 37°C, the plates were examined for presence of *Salmonella* serovar Enteritidis. Results from the two days were combined and statistics run on those combined results. In trial 2, 10 flies were collected from the room containing experiment-3 hens, day 3 post challenge, and placed into a tube containing1 ml PBS/0.5% tween 20 (Sigma Chemical Co., St. Louis, MO). The tube was vortexed for 30 s and the PBS/0.5% tween20 was transferred to 9 ml RV broth (=Exterior Wash). The fly carcass was then added to 1 ml bleach (1000 ppm) and vortexed 30 s and allowed to sit for 14.5 min. The salivary gland, crop and gut posterior from the proventriculus from each fly were aseptically excised and placed *en mass* into 5 ml RV broth. All samples were incubated for 24 h at 37°C and then plated onto BGNN, the plates incubated a further 24 h at 37°C and examined for the presence of *Salmonella* serovar Enteritidis. In trial 3, 10 flies were collected from the room containing experiment-3 hens, day 3 post challenge, and frozen for 24 h. The individuals were thawed and placed into bleach (1000 ppm) for 15 min and then dipped into PBS to remove bleach. The salivary gland, the crop and then the gut were aseptically excised from each fly and placed into individual 5 ml RV broths for each tissue. The broths were incubated at 37°C for 24 h and then plated onto BGNN which were incubated a further 24 h at 37°C and examined for the presence of *Salmonella* serovar Enteritidis.

***Salmonella* serovar Enteritidis transmission via contaminated flies.** Two trials were conducted to examine transmission of *Salmonella* serovar Enteritidis to previously unchallenged hens. In trial 1, 200 flies were captured live from Experiment 2 hens at day 7 post challenge and released into a room containing 24 hens in which feed had been removed 3 days previously, a time point chosen due to previous observations of maximal immunosuppression (15). Intestinal shedding of *Salmonella* serovar Enteritidis by the hens was examined on days 2 and 9 following release of the flies by administering 0.5 ml of 0.5% pilocarpine solution (Sigma) intraperitoneally to each hen and collecting the intestinal secretions released over the next hour onto polystyrene trays placed directly beneath each cage (21). One ml of secretion was added to 9 ml of RV broth. Following 24 h incubation at 37°C, the broths were plated onto BGNN and subsequently examined for the presence of *Salmonella* serovar Enteritidis 24 h later. In trial 2, 16 hens (8 hens/room) were transferred to two separate rooms and allowed 7 days to acclimate. Feed was removed for 14 days from birds in one room and on day 3 of feed removal, hens in both rooms received 5 flies/bird,

administered using a Pipetteman P1000 (Rainen Instruments, Woburn, MA). The flies, retrieved on day 9 of experiment 3, were loaded, 5 flies/tip, into P1000 tips whose ends were clipped to widen the bore diameter and allow passage of the flies. The tips were inserted onto the pipettor and the flies were expelled down the esophagus of each hen. The hens were sampled for the presence of *Salmonella* serovar Enteritidis in the crop and intestinal tract. Feed was removed from the fed group of birds the night before the sampling. Intestinal shedding was determined by intraperitoneal pilocarpine administration as described above. Crop colonization was determined as described previously (22). Briefly, 20 cm of tubing (Tygon™ 1/8" ID, 3/16" OD, Fisher Scientific) attached to a 10 ml syringe was inserted down the esophagus into the crop and 5 ml of crop lavage solution (1M tris/glycine buffer with 0.25% tween 20, pH 7-8) was administered into the crop. The lavage solution, along with crop luminal contents, was aspirated immediately back into the syringe and aseptically transferred into a 15 ml conical tube. Undiluted intestinal and crop samples (100 µl) were spread-plated onto BGNN plates and 1 ml of each undiluted sample was added to 9 ml tetrathionate brilliant green broth. The intestinal samples were also diluted 1:10 and plated onto BGNN plates using the Autoplate 4000 automatic dilution/plating system. The plates and broth enrichments were incubated for 24 h at 37C, plates examined for *Salmonella* serovar Enteritidis growth and, for any samples negative for *Salmonella* serovar Enteritidis, the tetrathionate enrichment broths were plated onto BGNN and examined for *Salmonella* serovar Enteritidis presence the next day. Samples with no growth on the direct plating, but positive in the tetrathionate enrichment were given an arbitrary count of 9 (1 below the theoretical detection limit) and samples with no growth in either the direct plating or the tetrathionate enrichment were given a count of 0.

Statistical Analysis. Statistical analyses were performed using GraphPad Instat (GraphPad Software, Inc., San Diego, CA). Unpaired t-tests were performed on comparisons between *Salmonella* serovar Enteritidis recovery from fly exteriors and interiors in trial 1 and the comparisons of chicken crop vs intestinal colonization by the organism following administration of contaminated flies. Analysis of variance (ANOVA) with Tukey's multiple comparison test procedures were conducted for *Salmonella* serovar Enteritidis recovery from the different organs within the fly (trial 3).

RESULTS

Fly contamination. Fly densities in the rooms, at the time of hen challenge, approached 200 flies/m^3

which would be considered low to moderate numbers in commercial egg operations (J.J Arends, Adjunct Professor, North Carolina State University Department of Entomology, personal communication). Within 48 h, 45-50% of flies in experiments 1 and 2 possessed detectable *Salmonella* serovar Enteritidis and recovery of the organism remained at 50% or greater for the next 5 days (Fig. 1). The numbers of *Salmonella* serovar Enteritidis residing in/on the flies ranged from 9 to 10^4. In experiment 3, 55% of flies were *Salmonella* serovar Enteritidis positive 24 hr post challenge which increased to 100% by day 4 and decreased thereafter (Fig. 1).

Fecal *Salmonella* serovar Enteritidis. Considerable fecal matter accumulated on the floors beneath cages over time. Levels of *Salmonella* serovar Enteritidis ranged from 10^3-10^7 *Salmonella* serovar Enteritidis /g feces the first week post challenge which decreased to 10^3-10^5/g by the end of the second week in both experiments (Fig. 2).

***Salmonella* serovar Enteritidis localization on/in the fly.** In trial 1, only a small percentage of flies possessed any detectable exterior *Salmonella* serovar Enteritidis, as indicated by culture results from the RV wash of each fly carcass. Conversely, a higher percentage ($P<0.05$) of the fly interiors contained the organism (Fig. 3). Repeat experiments using an RV wash or PBS showed similar results. This surprising observation called into question the efficacy using a simple aqueous wash to break the interaction between the *Salmonella* serovar Enteritidis and the fly. Using a PBS wash solution supplemented with 0.5% tween 20, a nonionic detergent, resulted in a dramatic increase in recovery of *Salmonella* serovar Enteritidis from the fly exterior (Fig. 3), equaling the recovery obtained from culturing the fly interior, 80-90%. In an effort to determine where the *Salmonella* serovar Enteritidis resided within the fly, salivary glands, crops, and guts were individually cultured and the results are shown in Table 1. No *Salmonella* serovar Enteritidis could be detected in fly salivary glands and only 10-20% of fly crops had detectable organism. The *Salmonella* serovar Enteritidis was recovered from all of the fly guts ($P<0.001$ vs salivary gland and crop).

S. enteritidis transmission via contaminated flies. To determine whether flies possessing *Salmonella* serovar Enteritidis could transmit the organism to naive hens, a study was conducted where contaminated flies were released into a room containing fasted naive hens. Infection of hens by *Salmonella* serovar Enteritidis via exposure to contaminated flies was not observed (data not shown). To determine whether *Salmonella* serovar Enteritidis from contaminated flies would be capable of colonizing hens, a second study was conducted where the birds were administered contaminated flies *per os* and then followed for shedding

of the organism over the next 3 weeks. An additional parameter, feed withdrawal, was assigned for one group as this was shown previously to dramatically increase susceptibility to infection (17). As shown in Table 2, minimal colonization of the chicken crop could be observed while intestinal colonization occurred in approximately 38% of the birds at day 6 ($P<0.05$ vs 0% in crop samples from fed hens) and 13 post challenge which dropped to 13-14% at day 20.

DISCUSSION

Flies from environments contaminated with human pathogens readily become contaminated themselves. Rosef and Kapperud showed in 1983 that 51% and 43% of flies captured on chicken farms and piggeries, respectively, in Norway were positive for *Campylobacter* (31). Bailey *et al*, were able to isolate *Salmonella* from 19% of flies captured on broilers farms (2) while Olsen and Hammack (29) found a 22% carrier rate of *Salmonella*, including *Salmonella* serovar Enteritidis, in flies captured in facilities housing laying hens. Numerous other studies have reported similar results (10, 27, 37). To the authors' knowledge, no studies have followed the kinetics of colonization with human pathogens of flies exposed to infected animals and their environment. Greenberg *et al* (9) fed gnotobiotic house flies graded doses of *S. typhimurium* and found that the flies excreted the organism in their feces at 24 h post challenge. The percentage of *Salmonella* excretors was dramatically reduced by 96 h post challenge indicating that, for the flies to remain positive for *Salmonella* over time, they needed a re-exposure. Shane *et al* (32) showed that house flies housed within an isolator cabinet containing chickens fecally shedding *Campylobacter jejuni* became colonized with the organism at 5 days. The current study demonstrated that the common house fly can rapidly become contaminated with *Salmonella* serovar Enteritidis by residing in an environment housing hens challenged with that organism. This contamination occurred within 24-48 h post hen challenge and persisted for at least two weeks. As Greenberg *et al* (9) showed that a re-exposure was necessary for *Salmonella* to persist in the fly host, a similar re-exposure to the contaminated fecal matter was probably necessary to allow the long-term *Salmonella* serovar Enteritidis prevalence.

The initial study comparing the frequency of recovery of *Salmonella* serovar Enteritidis from the fly exterior vs the interior indicated that the organism could be much more readily recovered from the fly interior and that the exterior frequency of recovery was very low (Fig. 3). This finding was observed on repeat occasions (data not shown) and was counter to the general consensus regarding flies as vectors of

human pathogens via a transfer of the organism through simple contact. Insects possess a waxy cuticle covering their entire exterior which serves as a barrier to water loss and also renders them waterproof (26). Adding a lipid solvent such as a detergent dissolves the wax and eliminates the waterproof character of the insect. Indeed, when the detergent tween 20 was added to the fly rinse buffer in the current study, recovery of the *Salmonella* serovar Enteritidis from the fly exterior increased to levels matching that observed for the interior (Fig. 3). These results indicate that the interaction of *Salmonella* serovar Enteritidis with the fly exterior is more dynamic than simply sitting on the surface of the fly. Further, simple physical contact may not be the primary method of transfer of the organism to different surfaces in a house and other mechanisms may be more prevalent.

Different microbial pathogens can reside in the fly alimentary tract (4, 9, 32). Greenberg *et al* (9) showed that *Salmonella* serovar Typhimurium fed to flies will reside and multiply in the fly gut. A high percentage of gut samples were culture positive for *Salmonella* serovar Typhimurium while minimal *Salmonella* serovar Typhimurium recovery was observed for the crop. The results shown in Table 1 of the current study mirror those of Greenberg *et al* in that a high percentage, 100%, of the gut samples were positive for *Salmonella* serovar Enteritidis while only 15% of crops had detectable *Salmonella* serovar Enteritidis (compared with previously reported 89% and 11% for gut and crops, respectively (Greenberg *et al* 9)). These results indicate that crop regurgitation by the fly during food collection is probably not a major source of pathogen spread while defecation by the fly is a much better possibility.

The importance of carriage of different pathogens by flies lies in their ability to transmit the pathogen to susceptible hosts. Shane *et al* in 1985 (32) showed that house flies taken from isolation cabinets housing *Campylobacter*-infected and fecally shedding chickens and introduced into a second isolation cabinet containing naive chickens could transmit the *Campylobacter* to these recipient birds. In the current study, transmission of *Salmonella* serovar Enteritidis was attempted through the introduction of flies collected from rooms containing infected hens into a room containing naive hens. To increase the potential for transmission to occur, feed was withdrawn from the recipients, mimicking a layer industry management tool, induced molting, used to achieve multiple egg-laying cycles from aging flocks. Prior to January, 2006, withdrawal of feed until the flock dropped 30% body weight, 10-14 d, was the primary method for flock recycling. This methodology has since been replaced in the U.S. with less-stressful diet regimens (35) while in Latin America and the Far East, representing greater than 50% of egg production

world-wide, feed withdrawal remains the primary method for molting flocks. Earlier studies have shown that feed withdrawal depressed cellular immunity in the birds (15, 16), resulting in increased severity of infection by *Salmonella* serovar Enteritidis (17, 18, 19, 20, 21) and dramatically increased the susceptibility of hens to infection (17). In the present study, this increased susceptibility had no apparent impact on the transmission of *Salmonella* serovar Enteritidis to the recipient birds, as no birds were found to be colonized by the organism. Several possible explanations for the discrepancy may be present. *Campylobacter* and S. enteritidis are entirely different organisms and transmission capabilities may therefore differ. Also, while birds in both studies received equivalent numbers of flies, the birds in the earlier study were housed in a relatively small contained space, a Horsfall isolation cabinet (0.6 m X 0.6 M X 0.6 M), while the hens in the current study were housed in a 5 m X 3 m X 3 m animal room. This latter situation dramatically expands the space that the flies may traverse, eliminates the intimate interaction brought on by small enclosed spaces, and decreases the fly density from 1000 flies/m^3 to 5 flies/m^3. However, *Salmonella* serovar Enteritidis-contaminated flies can transmit the organism when they are fed to naive hens (Table 2), indicating that transmission of *Salmonella* serovar Enteritidis by introducing contaminated flies into a room containing naive hens may be possible if a larger number of contaminated flies were used in the study or conversely, housing recipient birds in isolation cabinets to enhance contact between fly and bird. It should be noted that the number of flies released in this study was relatively low and represented a density substantially below what is commonly observed in commercial flocks.

The chicken crop is an enlargement or outpouching of the esophagus just proximal to the proventriculus or glandular stomach. The primary function of the crop is food storage while the stomach is full. The chicken crop was shown to routinely harbor *Salmonella* and was touted as an important source of broiler carcass contamination during processing (3, 5, 11). Birds challenged with *Salmonella* serovar Enteritidis will show equal colonization of the crop and intestinal tract (23). Interestingly, few of the crops from the recipient hens were culture positive for *Salmonella* serovar Enteritidis. Why these birds differ from those in previous studies remains to be determined. This may simply be a function of bacterial density, in that most challenge studies use 10^4-10^6 challenge doses while the flies in the current study generally carried much fewer organisms (range 9-10^4 organisms). Further, the crop tissues are bathed in organism following oral challenge while the fly-derived *Salmonella* would be trapped in and on the fly and just transits on through the organ. In the field broiler (5, 11) studies, the birds spent their lifespan on

contaminated poultry litter and, as a certain degree of coprophagy occurs during broiler grow-out, the crop will receive a regular re-contamination and re-colonization.

The present study reinforces what has been known for a long time, that flies residing in a contaminated environment will, themselves, become contaminated. However, to the authors' knowledge, the rapidity of contamination the flies and the localization of the organism within the individual insect following exposure to a contaminated environment has not been previously demonstrated. The mode of transmission via the fly vector appears to be a somewhat more complex situation, requiring more than just walking across a counter or landing on an exposed foodstuff. As contaminated flies administered to hens will initiate an infection, residing on or in a fly does not eliminate the infectivity of the *Salmonella* serovar Enteritidis organism. Although we did not observe direct transmission by releasing infected flies into a room with healthy birds, the number of flies used in the challenge was modest and probably below the threshold levels necessary to result in effective transmission of *Salmonella* serovar Enteritidis from flies to poultry. Additional work is needed with higher fly challenge densities and to determine the relative roles of fly ingestion and contamination of foods by fly fecal and vomit deposits. The mechanics and parameters of pathogen spread by flies still require a more in depth analysis before this "simple" phenomenon is understood.

ACKNOWLEDGMENTS

The authors would like to thank the excellent technical assistance of Dr. Lara Vaughn and Ms. Joyce Jacks during the performance of the studies. Thanks also to Dr. Lisa Durso, USDA/ARS MARC, Clay Center, NE, for the suggestion of including detergent in the fly rinse to release organisms adherent to the fly surface. A special thanks is extended to Dr. J.J. Arends, Adjunct Professor, Department of Entomology, North Carolina State University, Raleigh, NC, for sharing his expertise on fly levels found in commercial layer operations.

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Figure Legends

1. Recovery of *Salmonella* serovar Enteritidis (SE) from flies captured in rooms containing infected hens. Results represent the percentage of flies that were *Salmonella* serovar Enteritidis + on each sampling day post challenge in Expt. 1, 2, and 3. n = 20 flies/sampling day. * = flies sampled on day 9 post challenge in Expt. 3.

2. Concentration of *Salmonella* serovar Enteritidis (SE) in feces that collected under cages from infected hens over time post challenge. Results represent the Log_{10} *Salmonella* serovar Enteritidis /gram feces at the sampling times noted in Expt. 1 and Expt. 2. n = 4 samples/time period. ND = not determined

3. Simple aqueous rinsing does not remove *Salmonella* serovar Enteritidis from fly exteriors. Results represent the percentage of flies that were *Salmonella* serovar Enteritidis + for each sample type in Trial 1 (n = 14 flies/sample type) and Trial 2 (n = 10 flies/sample type). Number in parentheses = statistical difference between *Salmonella* serovar Enteritidis recovery from fly interior and exterior.

Table 1. Location of *Salmonella* serovar Enteritidis in the digestive tract of flies retrieved from rooms contaminated with *Salmonella* serovar Enteritidis [a]

Fly#	Salivary gland[b]	Crop	Gut
1	-	-	+
2	-	-	+
3	-	-	+
4	-	-	+
5	-	-	+
6	-	-	+
7	-	-	+
8	-	-	+
9	-	-	+
10	-	+	+
+/total	0/10	1/10	10/10 (P<0.001 vs crop & S. gland)

[a] Results represent individual flies recovered from rooms housing hens challenged with *Salmonella* serovar Enteritidis, day 3 post challenge.

[b] Salivary gland, crop, then gut were aseptically dissected out from exteriorly disinfected individual flies and cultured. Results represent the presence (+) or absence (-) of *Salmonella* serovar Enteritidis from each tissue from each fly.

Table 2. Initiation of *Salmonella* serovar Enteritidis infection in fasted vs nonfasted hens by oral administration of flies contaminated with *Salmonella* serovar Enteritidis [a]

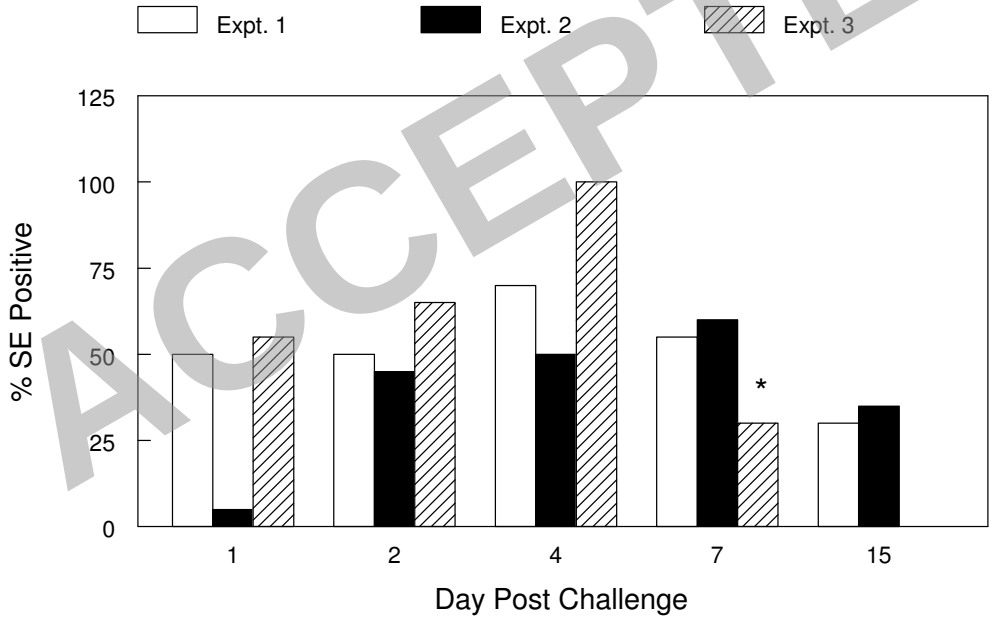
	% *S. enteritidis* +					
	Fed hens		*P* (crop vs intestine)	Fasted hens		*P* (crop vs intestine)
Day post Challenge	Crop[b]	Intestine		Crop	Intestine	
6	0	38	<0.05	13	38	NS[c]
12	0	25	NS	13	29	NS
20	0	13	NS	0	14	NS

[a] Results represent the percentage of crop and fecal samples that were *Salmonella* serovar Enteritidis positive on the days post challenge.

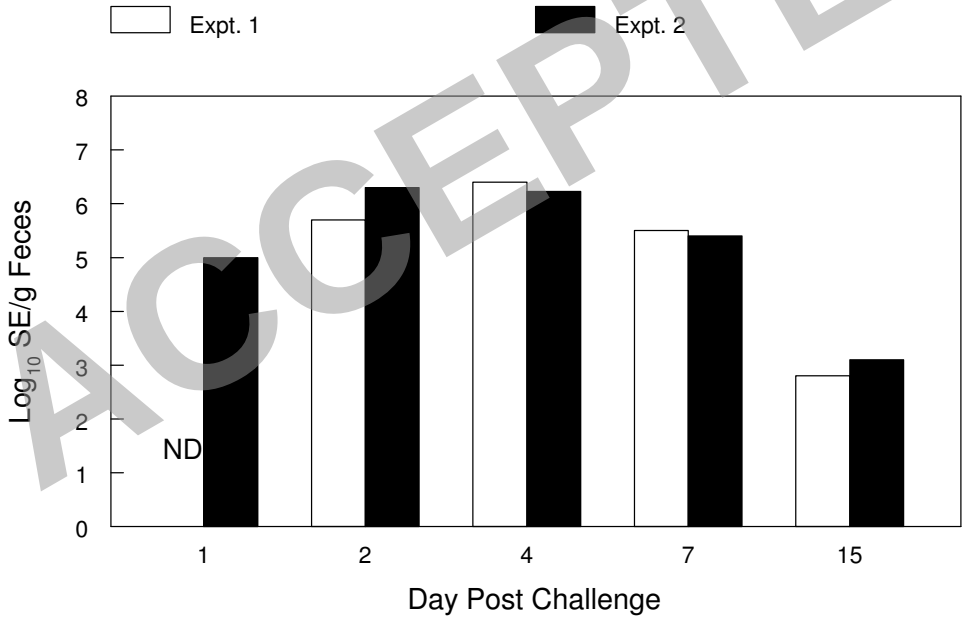
[b] Fed and fasted hens were orally administered flies contaminated with *Salmonella* serovar Enteritidis and the presence of the organism in crop and fecal samples was followed over time.

[c] Not Significant

Holt *et al.* Fig. 1



Holt *et al.* Fig. 2



Holt *et al.* Fig. 3